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13. Abstract (Maximum 200 Words) <i>(abstract should contain no proprietary or confidential information)</i> Reconstituted high density lipoprotein (rHDL) particles have been developed to incorporate water insoluble drugs to serve as drug delivery agents. Specifically, we have prepared taxol (TX) containing rHDL particles that are stable on rechromatography and preparative ultracentrifugation as no losses of TX from the complexes were seen during these studies. The rHDL/ TX complexes were as toxic to breast cancer cells as the commercial cremophor/taxol preparations. Taxol was avidly taken up by cancer cells from the rHDL/TX complexes, apparently by an SRB1 type receptor mediated mechanism. The uptake of core components from the rHDL complexes by a receptor mediated mechanism is supported by the observed expression of the SRB1 type receptors (demonstrated by immunoblotting) and by competition studies. Cancer cells were shown to exhibit considerably higher SRB1 receptor expression than fibroblasts, suggesting that the rHDL drug delivery system may be specifically targeting malignant cells.				
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Table of Contents

Cover.....	i
SF 298.....	ii
Table of Contents.....	iii
Introduction.....	1
Key Research Accomplishments.....	1
Reportable Outcomes.....	2
Conclusions.....	2
References.....	2
Appendices.....	3

INTRODUCTION

Adjuvant cancer chemotherapy has long been the accepted strategy for the treatment of several types of malignant tumors, especially those involving metastases (1). To enhance the efficacy of chemotherapy, multiple agents, longer and more intensive treatment, and combination therapy have been used. Although these modified approaches have resulted in improvements, the severity of side effects (2) and the gradual resistance developed by tumors during therapy (3) continue to hinder the efficacy of cancer chemotherapy. Consequently, more efficient delivery systems are needed to reduce the toxicity associated with cancer chemotherapy, while preserving the effectiveness of the respective chemotherapeutic agents.

In order to reduce the toxicity of chemotherapeutic agents, delivery vehicles including liposomes have been investigated (4,5). Some of the data emerging from these studies have shown that the efficacy of the drug delivery by liposomes was inversely related to the diameter of the particle (6). However, even the smallest liposomes have a diameter five times larger than the average high density lipoprotein (HDL) particle, a hydrophobic lipid micelle core with surface components of phospholipid and apolipoproteins (7). Because the overall properties of HDL are consistent with a potentially superior hydrophobic drug transporter, we have undertaken the investigation of rHDL particles as delivery vehicles for cancer chemotherapeutic agents.

KEY RESEARCH ACCOMPLISHMENTS

This is a "Concept Award" report. Due to the limited funding, the accomplishments are correspondingly modest. Nevertheless, we are pleased to report numerous exciting findings, two poster presentations at national meetings and some extended funding for this project.

The following scientific accomplishments can be documented during the second year of this award.

1. Studies using mice were conducted to investigate the stability of the rHDL preparations in vivo. These studies show that about ten times of the amount of the anti-cancer drug (taxol) is present in the circulation when it is administered as part of the rHDL complex as compared to the free drug
2. A model compound, dilauroyl fluoresceine, has been developed for the investigation of drug delivery to tumors ,using lipoprotein vehicles
3. Results of uptake studies, using transformed cell lines expressing high levels of the SRB-1 receptor are consistent with our earlier findings, indicating an important role for this receptor in the biology of cancer cells and the uptake of anti-cancer drugs.
4. Studies with an alternate drug delivery system, containing HDL components show that the uptake of anti-cancer drugs is substantially enhanced by the prsence of apolipoprotein-AI, the primary protein component of HDL. These findings suggest that the uptake of drugs,

using this mechanism may be able to overcome the tendency of many cancer cells and tumors for drug resistance.

Studies in Progress:

We are currently investigating new agents that may be particularly effective against breast cancer cells and tumors when encapsulated into the rHDL complex.

REPORTABLE OUTCOMES

We have had an article published in "Anticancer Research" (see attached).

We presented our findings at the "ERA of Hope" meeting in September, 2002.

We presented our findings at the ASBMB meeting in San Diego in April 2003.

We have obtained additional pilot funding to continue this project from the Faculty Research Fund.

We have filed an application with the US Patent Office.

CONCLUSIONS

We have completed all the projected goals that were outlined for this project in the "Concept proposal". The next phase of our studies involves translational studies with animals that will require substantially higher level of funding. Accordingly, we have been submitting proposals to the DOD Breast Cancer Program, the National Institutes of Health and to the Susan G. Komen Breast Cancer Foundation. We are hopeful that these efforts will result in additional funding and the continuation of our work.

Regarding the overall potential impact of our research, we believe that it has unlimited potential. Accordingly, our research has wide applications in breast cancer chemotherapy because, in addition to taxol, many of the frequently employed drugs are poorly water soluble and their application would thus be substantially improved by the rHDL delivery system. In addition, because of the vast potential for targeting, via modifications of its protein or lipid components, the future application of the rHDL drug delivery system could revolutionize chemotherapy via tumor specific targeting and potentially overcoming drug resistance. The proposed approach has the additional potential to substantially improve the delivery of anticancer drugs to hormone resistant breast tumors and thus enhance the prognosis for the survival of breast cancer patients.

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APPENDICES:

Paper published in *Anticancer Research*.

Abstract presented at the ERA of Hope meeting

Abstract presented at the ASBMB 2003 meeting.

High Density Lipoprotein Complexes as Delivery Vehicles for Anticancer Drugs

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Abstract. *Materials and Methods:* Recombinant high density lipoprotein (rHDL) particles were prepared with defined composition (phosphatidylcholine, apolipoprotein A-1, cholesterol and cholesteryl esters) and a molecular weight of approximately 187,000 kdaltons. Three molecules of taxol per rHDL particle were incorporated into these rHDL complexes. *Results:* Cholesteryl ester and taxol (HDL core components,) were taken up efficiently by several cancer cell lines compared to transformed normal ovarian cells (HGL5) used as the control. Immunoblotting with the scavenger receptor (SR-B1) antibody revealed strong immunoreactivity of several cancer cell lines. *Conclusion:* Our studies indicated that rHDL complexes can be used as efficient drug delivery vehicles due to the ability of cancer cells to acquire HDL core components. Targeting of anticancer drugs as components of the rHDL complex could result in reduction of toxic side-effects during chemotherapy.

Adjuvant cancer chemotherapy has long been the accepted strategy for the treatment of several types of malignant tumors, especially those involving metastases (1). To enhance the efficacy of chemotherapy, multiple agents, longer and more intensive treatment, and combination therapy have been used. Although these modified approaches have resulted in improvements, the severity of side effects (2) and the gradual resistance developed by tumors during therapy (3) continue to hinder the efficacy of cancer chemotherapy. Consequently, more efficient delivery systems are needed to reduce the toxicity associated with cancer chemotherapy, while preserving the effectiveness of the respective chemotherapeutic agents.

In order to reduce the toxicity of chemotherapeutic agents, delivery vehicles including liposomes have been investigated

(4,5). Some of the data emerging from these studies have shown that the efficacy of the drug delivery by liposomes was inversely-related to the diameter of the particle (6). However, even the smallest liposomes have a diameter five-times larger than the average high density lipoprotein (HDL) particle, a hydrophobic lipid micelle with surface components of phospholipid and apolipoproteins (7). Because the overall properties of HDL are consistent with a potentially superior hydrophobic drug transporter, we have undertaken the investigation of rHDL particles as delivery vehicles for cancer chemotherapeutic agents.

While preparations of reconstituted HDL were developed over thirty years ago (8), this approach has been used primarily to characterize the physical and chemical properties of HDL (9-11) and to conduct metabolic studies (12-13). Trials aimed at a clinical application of HDL particles have been reported to relieve endotoxic shock (14) and to arrest or reverse atherosclerosis (15). An application involving reconstituted HDL has been reported by Bijsterbosch and Van Berkel (16) who used lactosylated HDL to target delivery of antiviral drugs to liver tissue. Earlier studies also indicated that the proliferation of breast cancer cells and other adenocarcinoma cells (17-19) are dependent on the uptake of HDL components. Thus an additional advantage of using HDL for delivering chemotherapeutic agents is the direct uptake of hydrophobic core components from HDL by specific cell surface receptors, including those expressed by tumor cells (20,21).

The present study shows that a number of cultured human cancer cells are nearly as efficient as the model ovarian granulosa cells (HGL5) in the uptake of cholesteryl esters and taxol from the lipid core of recombinant HDL (rHDL) complexes, indicating that HDL may be used to solubilize otherwise very poorly water soluble (hydrophobic) therapeutic agents for injection into the systemic circulation.

Materials and Methods

Preparation of rHDL/drug complexes. Egg yolk phosphatidyl choline [Sigma] (1.8 mg in chloroform), 0.45 mg of unesterified cholesterol and 0.9 mg of cholesteryl oleate, both from Sigma, were dissolved in absolute alcohol, 300 µg of taxol (Mead Johnson) combined in a 15 ml conical

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Key Words: Lipoprotein complexes, anticancer drug delivery.

glass tube and the solvent was evaporated under N₂. Next, 5 ml of buffer (10 mM Tris.HCl pH 8.0, 0.15 M NaCl, 1 mM EDTA 0.025% NaN₃) was added. The mixture was sonicated in a Branson sonicator using a microtip, under the stream of N₂ at 60°C for 15 minutes and then stopped for 5 minutes. This cycle was repeated 4 times. The temperature was then lowered to 40°C and 10 mg of apoA-I (ZLB Bioplasma Inc., Berne Switzerland) was dissolved in 4 M guanidine HCl and then added slowly (in 10 minutes) to the lipid dispersion. Sonication was resumed for another 30 minutes at 40°C under N₂. The TX containing rHDL fractions were isolated by density gradient ultracentrifugation as described previously (22) and dialyzed overnight against 4.3 mM Na₂HPO₄, 1.2 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4 at 4°C.

Determination of rHDL components. Total cholesterol (Data Medical Associates, Arlington TX, USA), free cholesterol and phospholipids (Wako Pure Chemical Industries Ltd), were determined by respective enzymatic reagent kits, using microtiterplate assays (22). The concentration of cholesteryl esters was obtained as the difference between total and free cholesterol values. Taxol was determined by immunoassay as described by Leu *et al* (23). Protein determinations were carried out using Bradford reagent kits supplied by BioRad Laboratories.

Molecular weight determination. The molecular weight of the rHDL complex was determined by gel chromatography on 1.5X 80 cm AcA-34 (BioSeptra/Life Technologies Inc.) columns using apoferritin, alcohol dehydrogenase, serum albumin and ovalbumin as standards. The molecular weight of the rHDL complex was calculated based on the principles described by Ackers (24).

Cell cultures. The established cancer lines were obtained from the ATCC and grown according to procedures provided by the ATCC, employing the respective recommended media, including 1% penicillin and streptomycin mixture and 10% fetal bovine serum (Gibco/Life Technologies). HGL5 cells (25), gift of Dr. William H. Rainey of Southwestern Medical Center, Dallas TX, USA, were grown in DMEM F-12 (Gibco) with 1% ITS (BD Bio Sciences) and 10% FBS (Sigma). Cells were grown in 25-cm² flasks and split, using 0.25% trypsin to release the cells from the flasks, once 80-90% confluency was reached.

Monitoring of the uptake of core components from rHDL by cultured cells. On the first day the cells were plated in 12-well plates (50,000 cells/well) in their respective media. On the next day, the monolayers were washed with PBS pH 7.4 and then incubated at 37°C for 2 hours with varying amounts of rHDL-³H-cholesteryl oleate and rHDL-Taxol (¹⁴C-taxol) complexes in serum free media (KGM). Following the 2-hour incubation period, the cells were washed with PBS pH 7.4 and dissolved in 0.5M NaOH in preparation for scintillation counting.

Screening of cell cultures for taxol uptake. Radio-labeled ¹⁴C-taxol (Sigma) was incorporated into the rHDL particles along with the unlabeled taxol, as described above. The amount of TX taken up by the cells was monitored by scintillation counting. Taxol mass was computed from the specific radioactivities of the preparations that was earlier established by immunoassay (23).

Assessment of the cell killing potential of the rHDL/drug complexes. The cytotoxic effect of the rHDL/drug preparations on cancer cells was assessed by sulforhodamine B (26). The assessment of TX toxicity was performed as follows. Cells grown to 80-90% confluency were detached from the flask by digesting with 0.25% Trypsin. The cells were subsequently washed from the flask with complete media and centrifuged for 5 minutes at 1000g to pellet them. The cells were then resuspended in complete media and an aliquot was diluted 1:1 with Trypan Blue. The cells were counted and their viability determined as follows: the cells seeded in 96-well microtiter plates (at 3000 cells per

Table I. Composition of taxol containing rHDL preparations.

Component	Mean weight (μg) per ml (n=4)	% Distribution	μMoles per ml	Moles per MW=187,000
Lecithin	428±48	26	0.56	51
Cholesterol	9±0.9	0.6	0.23	3
Cholesteryl Oleate	24±1.6	1.5	0.035	4
ApoAI	1140±99	70.5	0.042	5
Taxol	17±1.9	1.1	0.02	3

*per MW=187,000

well) and allowed to attach for 24 hours. Taxol (Mead Johnson) was diluted to yield stock solutions of 10 nM to 100 nM and added in 50μl aliquots to each of the appropriate wells (total volume = 150 μl/Well). Controls included media + TX. Following a 24-hour exposure to TX and TX/rHDL complexes respectively, the medium was removed and the cells were fixed with 100μl of 10% trichloro acetic acid for 1 hour at 4°C. Subsequently, the samples were stained by 0.4% sulforhodamine B in 1% acetic acid. The stained protein was solubilized with 10mM Tris base and absorbance values were measured at 540 nm using a Bio-Rad microtiterplate reader.

Western-blot analysis. The cancer cells were grown until confluent, washed with PBS (pH 7.4) and scraped off the surface with 500μl of buffer containing 50mM Tris-HCl, 150mM NaCl, 0.02% sodium azide, 1% Triton X-100, 100μg/ml PMSF and 1μg/ml aprotinin. The cells were subsequently boiled for 5 minutes and then centrifuged at 1,000 rpm for 10 minutes and the supernatant used for Western-blot analysis. Ten μg of protein was mixed with equal volume of sample buffer, boiled for 4 minutes, and applied to a SDS-PAGE gel (10%). The protein was transferred to nitrocellulose overnight at 4°C. Immunoreactive bands were detected with a polyclonal rabbit antiserum (Novus biologicals) raised against a peptide containing residues 496-509 from mouse SRB-1, known to react with the human SRB-1 analog, CLA-1. Horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma) was used as a secondary antibody and visualization of immunoreactive bands was performed using the ECL detection system (Amersham).

Results

Production and characterization of the rHDL / taxol complexes. Recombinant HDL complexes, containing taxol (rHDL/TX) were prepared and characterized as follows. Table I shows the composition of the taxol containing rHDL complexes. The molecular weight of these rHDL/TX complexes (187,000) was established by gel chromatography as described under Materials and Methods. No loss of radioactivity was observed when a ¹⁴C-taxol/rHDL preparation was rechromatographed on the same column, indicating that the rHDL preparation was stable and free from taxol leakage during processing. The molecular weight of the isolated rHDL appears somewhat large (187,000) for the amount of protein being present in the complex (~ 70%

Table II. Comparison of the uptake of labeled ^{14}C taxol and labeled ^3H cholesteryl oleate from rHDL complexes by selected cancer cell lines. Uptakes by the non-malignant ovarian granulosa cell line (HGL5) were used as the control values (100%).

Cell Line	Cellular uptake of core component (%)	
	^{14}C -Taxol	^3H -cholesteryl ester
HGL5	100	100
DU 145	67	75
MCF7	73	72
T47D	74	90
OV 1063	74	75
PC3	83	91

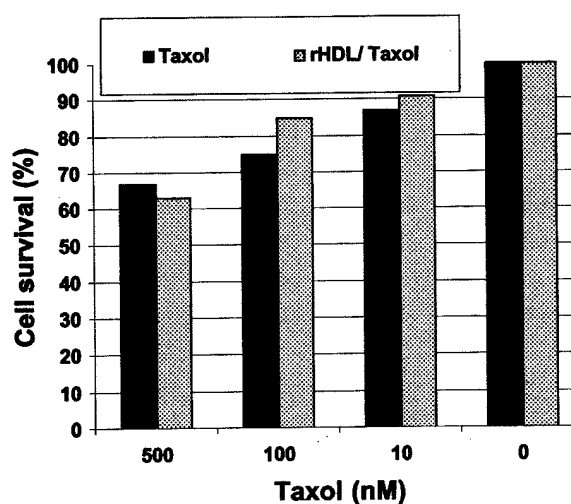


Figure 1. Comparison of the toxicity of taxol vs rHDL taxol preparations against PC-3 cells as monitored by the sulforhodamine dye assay.

by weight). The 3 moles of taxol per mole probably represents underloading of the rHDL/TX particles, as there is significant additional space in the core compartment, occupied by cholesteryl oleate (4% by weight).

The effectiveness of the rHDL / TX complex against cancer cells. The killing potential of the rHDL / drug complexes against tumor cells was assessed using the sulforhodamine B dye assay. Figure 1 shows the survival of PC-3 cells in the presence of taxol and rHDL/TX, respectively. The concentration of the drug was selected based on the studies of Liebmann *et al* (27). Similar studies were conducted with DU1 45, MCF7 and OV1 063 cell lines with essentially identical findings to those shown in Figure 1. These data show that the rHDL/TX complex is as efficient as TX in killing cancer cells even though the drug is apparently encapsulated by the HDL surface components.

The data in Table II show the uptake of HDL core components ^3H -cholesteryl oleate and taxol from rHDL by an ovarian granulosa cell line (HGL5) and by a series of cancer cells. The ovarian HGL5 cell line was used as the control in these studies to compare the ability of cancer cells to incorporate HDL core components to that of the assumed normal physiological target (20-21). The data show that all cancer cells were able to efficiently incorporate cholesteryl esters from rHDL (>65% of the model cell line). The correlation ($r=0.88$) between the efficiency of cholesteryl ester and taxol uptake by a variety of malignant cells suggest that cancer cells express an HDL receptor, perhaps of the SRB-1 type, that allows the selective uptake of core components from HDL.

Figure 2 shows the immunoblots of cell extracts obtained

with an SRB-1 antibody. Here the reactivity of the control ovarian cell line (HGL5) was compared with those of a number of cancer cells and a fibroblast cell line. The data show significant immunoreactivity of all cancer cells, especially when compared to normal cells (fibroblasts) that are not known to express this receptor (28).

Finally the specificity of the core component uptake from rHDL was examined by assessing the competition between ^3H -cholesteryl oleate labeled and unlabeled rHDL preparations. The data for two cancer cell lines show (Figure 3) that the unlabeled rHDL suppressed the uptake of the labeled cholesteryl ester to a larger extent in the cancer cells compared to the HGL5 ovarian granulosa cell line. These data suggest that the uptake of rHDL core components is at least as specific (mediated by the SRB-1 type receptors) as for ovarian tissue.

Discussion

Although improvement of cancer chemotherapy by the use of delivery vehicles has been impressive over the last several decades (4,5), important challenges, including toxicity of therapeutic agents and drug resistance have been encountered with several types of cancer. We undertook the study of rHDL complexes as potential delivery vehicles for chemotherapeutic agents because they appeared to have desirable properties as drug transporters, due to their size and the receptor mediated uptake of HDL core components (6,18,19). The latter feature could be particularly important as the rapidly dividing cancer cells are likely to have increased cholesterol requirements for membrane biogenesis. In fact, earlier studies have shown that the proliferation of

HGL5 T47D PC3 OV1063 MCF7 DU145 HeLA fibroblast



Figure 2. Immunoblot of the SRB-1 receptor in normal and cancer cell lines.

adenocarcinoma cells (20) and breast cancer cells (21) was dependent on the uptake of HDL or HDL components. More recently, at least one breast cancer cell line was shown to express active HDL receptors (29). Based on these findings, combined with reports in the literature (17-19), it is clear that HDL type particles may serve as highly efficient delivery vehicles of anticancer agents. Because of the relatively low levels of expression of these receptors in most peripheral tissues (23), it is likely that the drugs delivered via HDL type vehicles could have reduced toxic side effects compared to the free drug.

The application of rHDL particles as delivery vehicles for cancer chemotherapeutic agents may be advantageous for several reasons. Poor water solubility is a common feature of many pharmaceutical preparations including several chemotherapeutic agents (29,30). A number of recent efforts have been reported to overcome this problem and to reduce the toxicity of the intravenously administered taxol preparations (31,32). The rHDL/drug preparations may be particularly effective in this regard as the core components of HDL are normally made up of highly water insoluble compounds (triglycerides and cholesteryl esters). Concerning the effectiveness of taxol and other chemotherapeutic agents, the lack of tumor selectivity is another serious concern. The structure of rHDL allows for the alteration of lipid and protein composition and the covalent attachment of potential targeting vehicles to augment the selectivity of specific agents used in cancer chemotherapy.

Drug resistance of specific tumor cell lines is another major concern during cancer chemotherapy. In addition to the known drug resistance tumors, certain malignant cells can develop resistance to a particular agent during chemotherapy. The ability of tumors to become resistant to chemotherapy has been linked to specific plasma membrane components (33), including the transmembrane P-glycoprotein (34). Because the rHDL preparation is able to interact with specific cell surface receptors (28) and deliver its core components to the interior of the cells without endocytosis, this mechanism may be successful for delivering chemotherapeutic agents without inducing drug resistance.

In summary, we have prepared recombinant high density lipoprotein complexes (rHDL) that contain defined quantities of taxol. We have shown for the first time that the drug incorporated into the rHDL particles is

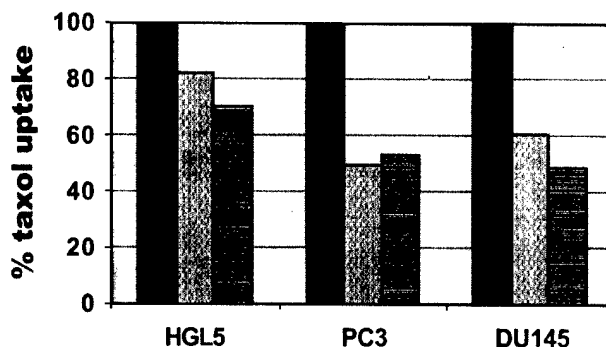


Figure 3. Comparison of the ^3H -cholesteryl ester uptake from rHDL preparations by ovarian granulosa (HGL5) and two cancer cell lines in the presence of half (dotted bars) and equal amounts of unlabeled rHDL (horizontally lined bars). The solid black bars represent the controls with no unlabeled HDL added. The data are presented as the percentage of the uptake by the HGL5 cells (100%).

efficiently taken up by several cancer cells presumably via a receptor mediated (SR-B1 type) mechanism. This drug delivery system may be effective in overcoming the poor solubility of many pharmaceutical preparations, increase the selectivity of anticancer drugs and possibly reduce the drug resistance against certain agents, encountered during chemotherapy.

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Reconstituted high density lipoprotein (rHDL) particles have been developed that are able to incorporate water insoluble drugs as core components and thus are excellent potential targeted drug delivery agents. Specifically, we have prepared taxol (TX) containing rHDL particles that exhibit the expected molecular weight, lipid and protein compositions. These rHDL/TX particles are stable on rechromatography and preparative ultracentrifugation as no losses of TX from the complexes were seen during these studies. The rHDL/ TX complexes were as toxic to breast cancer cells as the commercial cremophor/taxol preparations. Taxol was avidly taken up by cancer cells from the rHDL/TX complexes, apparently by an SRB1 type receptor mediated mechanism. The uptake of core components from the rHDL complexes by a receptor mediated mechanism is supported by the observed expression of the SRB1 type receptors (demonstrated by immunoblotting) and by competition studies. Cancer cells were shown to exhibit considerably higher SRB1 receptor expression than fibroblasts, suggesting that the rHDL drug delivery system may be specifically targeting malignant cells.

These studies show that the rHDL drug delivery system is effective in killing breast cancer cells while it may have reduced toxicity toward normal cells. The ability of the rHDL delivery vehicles to solubilize otherwise highly hydrophobic drugs (taxol) without the use of toxic solubilizing agents (cremophor) is an additional advantage of this system. The rHDL particles may also be able to overcome the drug resistance of many tumors because of the receptor mediated uptake of their core components.

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Targeted Drug Delivery By Reconstituted High Density Lipoproteins (rHDL).
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A new approach for drug delivery to malignant tumors has been developed by encapsulating the motherapeutic agents into reconstituted high density lipoproteins (rHDL). These rHDL complexes resemble normal HDL and they markedly improve the solubility and transportability of otherwise poorly soluble drugs. Studies using the hydrophobic anti-cancer agent taxol revealed that the rHDL/taxol complexes were as toxic to cancer cells as the commercial cremophor/taxol preparations. Taxol uptake by several cancer cells from the rHDL/TX complexes apparently occurred via an SR-BI type receptor mediated mechanism, as demonstrated by immunoblotting and by competition studies. Examination of the distribution of taxol injected into mice showed that the majority of the drug was rapidly taken up by the liver. However, over three times as much taxol remained in the blood when injected as a component of the rHDL complex, as compared to the free taxol. These data suggest that the rHDL drug delivery system is capable of extending the maximum plasma concentration in addition to facilitating the solubility of hydrophobic drugs. Further potential advantages of the new drug delivery system involve targeting via chemical modifications of the protein or lipid constituents of the rHDL complex, in addition to overcoming the resistance of certain tumors against anti-cancer drugs.